Chang Q *et al* / Acta Pharmacol Sin 2003 Aug; 24 (8): 796-804

Effects of chiral 3-*n*-butylphthalide on apoptosis induced by transient focal cerebral ischemia in rats[1]

CHANG Qing, WANG Xiao-Liang[2]

Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

KEY WORDS apoptosis; brain ischemia; 3-*n*-butylphthalide; cytochrome c; caspase-3

ABSTRACT

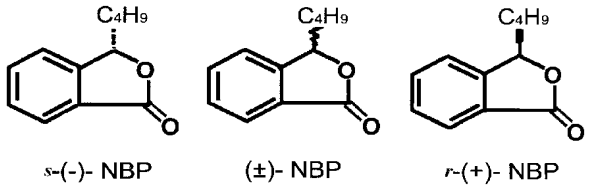
AIM: To investigate the effects of 3-*n*-butylphthalide (NBP) on apoptosis induced by transient focal cerebral ischemia in rats, compare the action potency of *s*-(-)-, *r*-(+)- and (±)-NBP, and clarify the enantiomer that played a main role. **METHODS:** DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay and gel electrophoresis. The expression of cytochrome c and caspase-3 protein was observed by Western blot analysis and immunohistochemistry. Middle cerebral artery was occluded for 2 h. **RESULTS:** Significant DNA fragmentation was detected at 24 h after reperfusion. This response was inhibited by *s*-(-)-NBP (5, 10 mg/kg ip). *s*-(-)-NBP 10 mg/kg almost completely inhibited DNA fragmentation, whereas *r*-(+)- NBP 10 mg/kg showed less effect. (±)-NBP (20 mg/kg) showed an inhibitory effect between that of *s*-(-)-NBP (10 mg/kg) and *r*-(+)-NBP (10 mg/kg). During the apoptotic process, cytochrome c was released into the cytosol and caspase-3 was activated. This effect was markedly inhibited by *s*-(-)-NBP, and the action potency of *r*-(+)- and (±)-NBP on the changes of cytochrome c and caspase-3 protein was similar to that on DNA fragmentation. **CONCLUSION:** NBP, especially its *s*-(-)-enantiomer, could potently reduce the release of cytochrome c, decrease the activation of caspase-3, and inhibit DNA fragmentation after transient focal cerebral ischemia. Our findings on the beneficial effects of NBP on cerebral ischemia-induced apoptosis might have important implications for the study and treatment of ischemic cerebrovascular diseases.

INTRODUCTION

s-(-)-3-*n*-Butylphthalide [*s*-(-)-NBP] was extracted as a pure component from seeds of *Apium graveolens* Linn[1]. Then, (±)-NBP was synthesized and developed as an anti-cerebral ischemic agent, recently, it has finished phase III clinical trial. There is a chiral center in the molecule of NBP, and a pair of enantiomers were synthesized.

NBP has been reported to have many anti-ischemic effects, including: reducing the area of cerebral infarct in middle cerebral artery occlusion (MCAO) rats[2], attenuating neuronal damage after delayed cerebral injury[3], ameliorating mitochondria dysfunction[4,5] during cerebral ischemia, improving energy metabolism in complete brain ischemic mouse[6], ameliorating brain edema and blood-brain barrier damage in MCAO rats[7,8], enhancing regional blood flow in MCAO and subarachnoid hemorrhage model[9,10], improving microcirculation in pial arterioles in MCAO rats[11], and delaying the life span and improving neurological deficit in stroke prone

[1] Project supported by the State Science and Technology Commission grant (No 94-ZD-01) and by the National Natural Science Foundation of China (No 29790122).
[2] Correspondence to Prof WANG Xiao-Liang.
Phn 86-10-6316-5193. Fax 86-10-6301-7757.
E-mail wangxL@imm.ac.cn
Received 2002-07-09 Accepted 2003-01-03



spontaneously hypertensive rats[12]. A number of studies have shown that neuronal cell loss after cerebral ischemia involved apoptosis. After focal cerebral ischemia in rats, the predominant localization of apoptotic cells at the inner boundary of the ischemic lesion suggested that the apoptotic process largely contributed to the expansion of the ischemic damage[13]. Moreover, several laboratories have demonstrated that antiapoptotic maneuvers could reduce neuronal death and infarct volume[14,15]. The effects of (±)-NBP on hypoxia/hypoglycemia-induced apoptosis of rat cortical neurons have been previously reported[16]. Based on the fact that the biological activities and toxicities of drugs are closely related to their optical activities, the present study was designed to compare the action potency of *s*-(-)-, *r*-(+)-, and (±)-NBP on cerebral ischemia-induced apoptosis and clarify the enantiomer that played a main role.

The apoptotic pathway involves at least three functional distinct phases: an initiation phase during which cells receive death stimuli; an effector phase dependent on bcl-2 family members and on apoptogenic proteins released from mitochondria; and a degradation phase, dependent on caspases. The participation of mitochondria in the mechanism of cell death has received much attention in recent years. Mitochondria is assumed to be involved in apoptosis by releasing apoptogenic proteins, such as cytochrome c, to the cytoplasm where it activates caspase-3, a cysteine protease of the IL-1β-converting enzyme family, which has been reported to trigger apoptosis[17,18]. The protective effects of NBP on ischemia-induced mitochondrial injury and mitochondrial morphological changes[4,5] have been previously reported and it was therefore of interest to investigate the effects of chiral NBP on the release of cytochrome c and on the activation of caspase-3 after transient focal cerebral ischemia.

MATERIALS AND METHODS

Drugs *s*-(-)-, *r*-(+)-, and (±)-NBP (purity >99 %) were supplied by Department of Medicinal Chemistry of our Institute. The drugs were prepared into emulsion with 0.5 % Tween-80 before use.

Focal cerebral ischemia The experimental protocol was approved by the committee of Chinese Academy of Medical Sciences on ethics of animal experimentation. Adult male Wistar rats weighing 270-300 g (Experimental Animal Center of Chinese Academy of Medical Sciences, Grade II, Certificate No 2000017) were subjected to transient focal ischemia by intraluminal middle cerebral artery (MCA) blockade with a nylon suture, as previously described[19]. Briefly, rats were anesthetized with 10 % chloral hydrate (350 mg/kg ip), the rectal temperature was maintained in the range of 36.5-37.5 °C, the bifurcation of the common carotid artery was exposed, the external carotid artery was dissected and coagulated distally, and the internal carotid artery was isolated and separated from the vagus nerve. A 40-mm length of monofilament nylon suture (Φ0.26 mm) was introduced into the internal carotid artery through the stump of the external carotid artery and gently advanced for a distance of 20 mm from the common carotid artery bifurcation to block the origin of the MCA. The MCA blood flow was restored by the withdrawal of the nylon suture 2 h after occlusion. Sham operations were performed using the same surgical procedures except that no suture was inserted. Animals were divided into seven groups (n=10 for each): 1) sham-operated animals; 2) vehicle treated animals: only 0.5 % Tween-80 was administered; 3) and 4) *s*-(-)-NBP 5 and 10 mg/kg-treated animals; 5 and 6) *r*-(+)-NBP 5 and 10 mg/kg-treated animals; 7) (±)-NBP 20 mg/kg treated animals. *s*-(-)-, *r*-(+)- and (±)-NBP were given ip 10 min after ischemia.

Neurological deficits The evaluation of neurological deficits was carried out at 24 h after reperfusion using the neurological grading system (grading of 0 to 3) developed by Bederson *et al*[20]. The rater was naive to the treatment protocol and to the identity of the groups.

The terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL) assay At 24 h after reperfusion, animals were reanesthetized and perfused with 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) followed by 4 % paraformaldehyde in PBS. The paraffin-embedded coronal sections (10 μm thick) at the level of the caudate putamen that showed typical infarction were selected and processed for TUNEL staining, according to the method of Gavrieli *et al*[21]. Briefly, sections were placed in TdT buffer for 15 min, followed by reaction with TdT

enzyme and biotinylated 11-dUTP at 37 °C for 60 min. Avidin-horseradish peroxidase solution was applied to the sections, and the staining was visualized with diaminobenzidine. Slides were counterstained with methyl green.

DNA laddering At 24 h after reperfusion, animals were reanesthetized and decapitated. The brains ipsilateral to the ischemic hemisphere were rapidly removed and homogenized in lysis buffer containing 0.5 % sodium dodecyl sulfate, Tris-HCl 10 mmol/L, pH 8.0, edetic acid 0.1 mol/L, proteinase K (Merck) 100 mg/L and incubated in the same buffer for 3 h at 50 °C. After digestion, samples were treated with 20 mg/L DNA-free RNase (Sigma) at 37 °C for 1 h. Then DNA was extracted with equal volumes of phenol and phenol-chloroform-isoamyl alcohol (25:24:1), and precipitated for 2 h with 0.3 mol/L sodium acetate, pH 5.2 and two volumes of 100 % ethanol at -20 °C. The DNA was washed with 70 % ethanol, air dried, and resuspended. Gel electrophoresis for detecting DNA laddering was performed with the TACS™ Apoptotic DNA Laddering Kit (Trevigen), as previously described[22]. Before electrophoresis, the samples were reacted with Klenow enzyme (Trevigen) and dNTP (Trevigen). Then, samples were subjected to electrophoresis on a 1.5 % agarose gel. After that, the gel was washed and DNA was transferred to a nylon membrane. The membrane was first blocked by 5 % powdered milk (Sigma), then incubated with streptavidin-horseradish peroxidase conjugate (Trevigen). Finally, the bands were visualized by chemiluminescence method using PeroxyGlow (Trevigen), and the films were exposed to X-ray film.

Isolation of total proteins, and cytosolic and mitochondrial protein fractions At 3, 6, 12, 24 and 72 h after reperfusion, animals were reanesthetized and ipsilateral hemispheric brains were obtained. Homogenization was performed in ice-cold buffer [Tris 50 mmol/L, pH 7.4, NaCl 150 mmol/L, 0.5 % Triton X-100, edetic acid 1 mmol/L, phenylmethylsulfonyl fluoride 1 mmol/L, and aprotinin 5 mg/L], and centrifuged at 12 000×*g* at 4 °C for 30 min. Total proteins present in the supernatant were then collected.

Protein extraction of both the mitochondrial and the cytosolic fractions was performed as described[22]. Briefly, sham-operated brain or ischemic brain was cut into pieces and gently homogenized by being dounced in a glass tissue grinder in cold suspension buffer (20 mmol/L HEPES-KOH, pH 7.5, sucrose 250 mmol/L, KCl 10 mmol/L, $MgCl_2$ 1.5 mmol/L, edetic acid 2 mmol/L, phenylmethylsulfonyl fluoride 0.1 mmol/L, aprotinin 2 mg/L and leupeptin 10 mg/L). The homogenates were centrifuged at 800×*g* for 15 min at 4 °C and then at 8000×*g* for 20 min at 4 °C. The 8000×*g* pellets were used to obtain the mitochondrial fraction. The supernatant was further centrifuged at 100 000×*g* for 60 min at 4 °C. Protein concentrations were determined by the Lowry method.

Western blot analysis Protein samples (10 mg) were electrophoresed through a 15 % sodium dodecyl sulfate polyacrylamide gel. After electrophoresis, proteins were electrically transferred to a nitrocellulose membrane at a constant current of 0.65 mA/cm^2 for 2 h. The membrane was incubated with blocking solution [5 % nonfat milk in TBST (Tris-HCl 20 mmol/L, pH 7.5, NaCl 150 mmol/L, and 0.05 % Tween-20)] at 4 °C overnight and subsequently incubated with primary antibodies diluted in TBST at room temperature for 2 h. Primary antibodies were either a mouse monoclonal antibody directed against cytochrome c (7H8.2C12, Pharmingen, 1:1000 final dilution) or a rabbit polyclonal antibody against caspase-3 (Santa Cruz Biotechnology, 1:200 final dilution), which recognized the unprocessed procaspase-3 (M_r=32000) and the p17 subunit (M_r=17000) of the active caspase-3. After washing with TBST, the membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG) at room temperature for 1 h. Immunoblots were visualized using enhanced chemiluminescence Western blotting detection reagents (Santa Cruz Biotechnology). The optical density (*OD*) of the corresponding Western blot band was measured using Kodak ID Image software. Data were calculated as % of *OD* levels of the vehicle group. Western blot analysis of β-actin was also performed.

Immunohistochemical analysis Rats were reanesthetized at 24 h after reperfusion. After transcardiac perfusion, brains were cryoprotected in 25 % sucrose in PBS and coronal brain sections (20 μm thick) were cut on a cryostat (Slee Medical Equipment Ltd, UK). An ABC Kit (Zhongshan Biotechnology, Beijing) was used to localize the primary antibody and a Diaminobenzidine Kit (Zhongshan Biotechnology, Beijing) was used to visualize the catalyzed peroxidase-reaction product. Sections were then counterstained with methyl green. The mouse monoclonal antibody against rat cytochrome c (7H8.2C12, Pharmingen) was used at a 1:500 dilution and the rabbit polyclonal antibody against

caspase-3 (the same antibody used for caspase-3 Western blot analysis) was used at a 1:100 dilution. As a negative control, sections were incubated without primary antibodies.

Statistical analysis Data were expressed as mean±SD (Student's *t*-test). For quantitative analysis of TUNEL-positive cells, coronal brain sections at the level of the anterior commissure (+0.5 mm from bregma) under high-power microscopic fields (×200) in two brain regions, parietal cortex and caudate putamen, were selected and quantitated. Microscopic fields for cell counting were chosen in areas where the maximal amounts of positively stained cells were present. Five microscopic fields per region per section were analyzed[29].

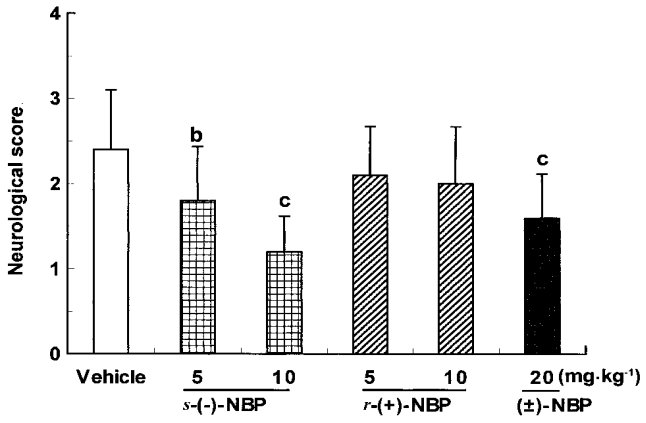


Fig 1. Effects of chiral NBP on neurological deficits in MCAO (2 h of ischemia and 24 h of reperfusion) rats. *n*=10. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle.

RESULTS

Effects of chiral NBP on neurologic deficits *s*-(-)-NBP at the doses of 5 and 10 mg/kg markedly inhibited the neurological deficit as compared to vehicle group ($P<0.05$), but *r*-(+)-NBP (5, 10 mg/kg) showed no such effect ($P>0.05$). (±)-NBP 20 mg/kg led to less reduction in the grading of neurologic deficit than *s*-(-)-NBP 10 mg/kg ($P<0.05$, Fig 1).

Effects of chiral NBP on DNA fragmentation According to the previous reports[13], DNA fragmentation peaked at 24-48 h of reperfusion after 2 h of MCAO, thus the time-point of 24 h after reperfusion was selected to study the effects of chiral NBP on DNA fragmentation, which were characterized by TUNEL analysis and DNA laddering.

TUNEL staining has been used extensively to identify cells with nuclear DNA fragmentation. Cells were scored as apoptosis when they were TUNEL-positive (brown staining) and showed nuclear chromatin condensation (arrows in Fig 2B). Only zero to three TUNEL-positive cells were observed in each

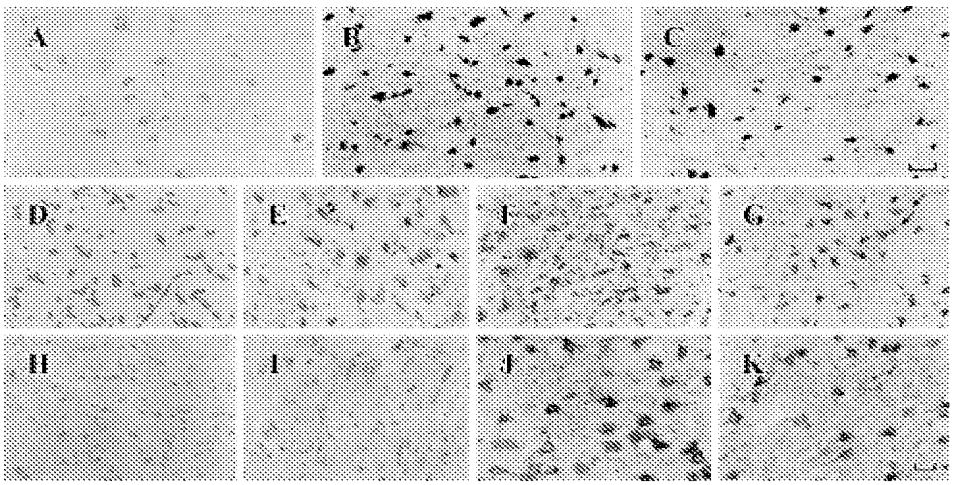


Fig 2. (A-C) Representative photomicrographs of TUNEL analysis of cells in parietal cortex in coronal brain sections at the level of the anterior commissure (+0.5 mm from bregma) in MCAO (2 h of ischemia and 24 h of reperfusion) rats treated with chiral NBP. (A) Sham. (B) Vehicle. Arrows: apoptotic bodies. Arrowheads: necrotic neurons. (C) *s*-(-)- NBP 10 mg/kg. (D-K) Representative photomicrographs of cytochrome c (D to G) and caspase-3 (H to K) immunostaining in parietal cortex in coronal brain sections at the level of the anterior commissure (+0.5 mm from bregma) in MCAO (2 h of ischemia and 24 h of reperfusion) rats treated with chiral NBP. (D, H) Sham. (E, I) Negative controls. Arrowheads in E: cells representing nuclear fragmentations. (F, J) Vehicle. (G, K) *s*-(-)-NBP 10 mg/kg. Arrows in G: axon-like long processes. *n*=3. ×400. Bar=15 μm.

hemispheric section of the sham-operated rats and in the contralateral hemisphere of ischemic rats. These cells with nuclei not stained with TUNEL (TUNEL-negative, green-colored) were visualized by counterstaining with methyl green (Fig 2A). In contrast, in the ipsilateral hemisphere of the ischemic brain in the vehicle group, a large number of TUNEL-positive cells, which were mainly located in the parietal cortex (265±16) and in the inner boundary of the caudate putamen (387±21), were seen at 24 h after reperfusion (Fig 2B, Fig 3). *s*-(-)-NBP (5, 10 mg/kg) markedly reduced the number of TUNEL-positive cells compared with the vehicle group (Fig 2C, Fig 3, $P<0.05$). However, only the higher dose of *r*-(+)-NBP (10 mg/kg) showed a slight inhibitory effect in parietal cortex (Fig 3). Surprisingly, the inhibitory effect of (±)-NBP 20 mg/kg was less than that of *s*-(-)-NBP 10 mg/kg (Fig 3, $P<0.05$).

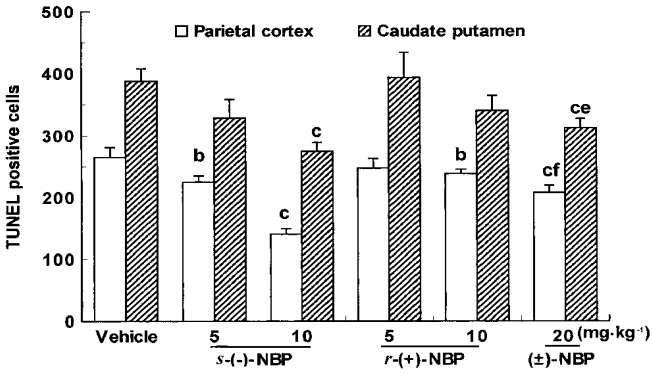


Fig 3. Quantitative evaluation of the effects of chiral NBP on the number of apoptotic cells in MCAO (2-h ischemia and 24-h reperfusion) rats. TUNEL-positive cells were quantified under high-power microscopic fields (×200) in two brain regions, parietal cortex and caudate putamen , in coronal brain sections at the level of the anterior commissure (+0.5 mm from bregma). Five microscopic fields per region per section were analyzed. n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* vehicle. $^eP<0.05$, $^fP<0.01$ *vs* *s*-(-)-NBP (10 mg/kg).

In addition to TUNEL analysis, we analyzed the internucleosomal cleavage of DNA by gel electrophoresis. No DNA laddering was seen in sham-operated rats (Fig 4, Lane 1), whereas a significant amount of DNA laddering was detected in vehicle-treated rats (Fig 4, Lane 2) at 24 h after reperfusion. DNA laddering was markedly inhibited in *s*-(-)-NBP (5, 10 mg/kg) treated rats (Fig 4, Lane 3, 4) compared with vehicle group. *s*-(-)-NBP 10 mg/kg almost completely inhibited DNA laddering, whereas *r*-(+)-NBP 10 mg/kg (Fig 4, Lane 6) showed weak effect. Interestingly, the inhibitory effect of (±)-NBP (20 mg/kg) (Fig 4, Lane 7) seemed to be in the range between that of *s*-(-)-NBP (10 mg/kg) and *r*-(+)-NBP (10 mg/kg).

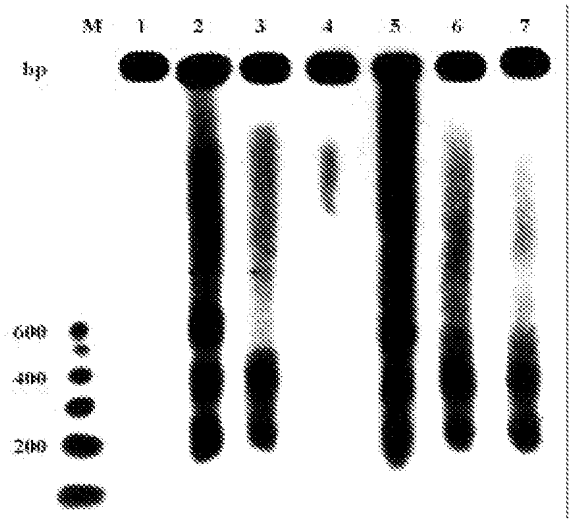


Fig 4. Effects of chiral NBP on DNA laddering induced by MCAO (2 h of ischemia and 24 h of reperfusion) in rat brain. The ladders corresponding to 200, 400, and 600 base pairs were shown. Lane M, DNA marker; Lane 1, sham; Lane 2, vehicle; Lane 3 and 4, *s*-(-)-NBP 5 and 10 mg/kg; Lane 5 and 6, *r*-(+)-NBP 5 and 10 mg/kg; Lane 7, (±)-NBP 20 mg/kg. n=4.

Effects of chiral NBP on the release of cytochrome c Cytochrome c immunoreactivity was obvious as a unique band (M_r=15000) detected by Western blot analysis. When a strong immunostaining was present in mitochondrial fractions of brain from sham-operated rats, no band was seen in the cytosolic fractions (Fig 5A to 5B, Lane 1). The immunoreactivity of the mitochondrial fractions was decreased as early as 3 h after reperfusion in ischemic brain, with a corresponding increase in cytosolic fractions (Fig 5A, Lane 2). The release of cytochrome c was found to be maximal at 24 h after reperfusion (Fig 5A, Lane 5) and the effects of chiral NBP were therefore evaluated at this time-point in subsequent studies. *s*-(-)-NBP (5, 10 mg/kg) (Fig 5B, Lane 3, 4) markedly inhibited the distributional change of cytochrome c, whereas *r*-(+)-NBP (5, 10 mg/kg) (Fig 5B, Lane5, 6) showed no effect (Fig 5C, $P<0.05$). Surprisingly, (±)-NBP 20 mg/kg (Fig 5B, Lane 7) showed less effect than *s*-(-)-NBP 10 mg/kg (Fig 5C). On the other hand, a consistent amount of β-actin immunoreactivity was seen in the bottom panel, suggesting that the amount of the loaded protein was con-

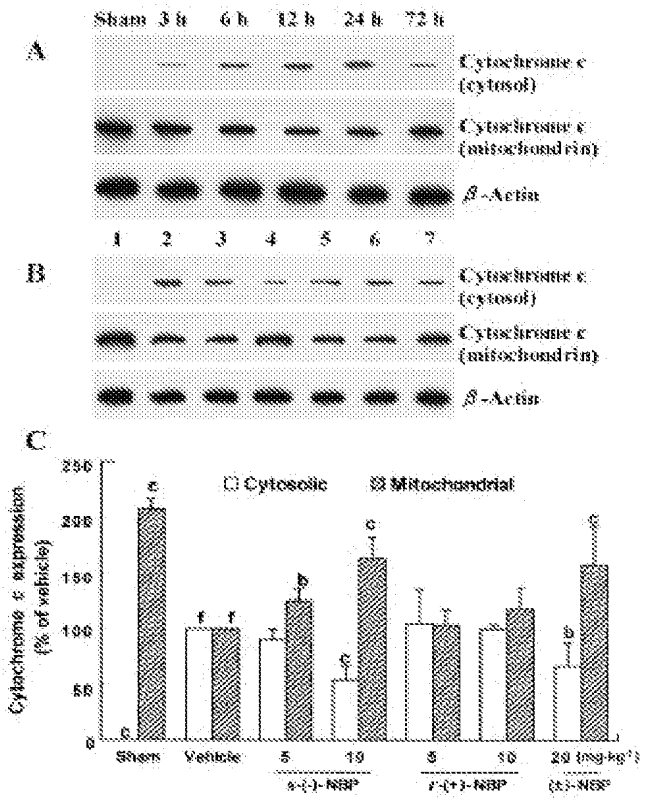


Fig 5. Western blot analysis of cytochrome c in cytosolic and mitochondrial fractions from MCAO rats with β-actin protein as an internal control. (A) Time-dependent release of cytochrome c from mitochondria into the cytosol during reperfusion after 2 h of MCAO. (B) Effects of chiral NBP on the levels of cytochrome c after 24-h reperfusion. Lane 1, sham; lane 2, vehicle; lane 3 and 4, *s*-(-)-NBP 5 and 10 mg/kg; lane 5 and 6, *r*-(+)-NBP 5 and 10 mg/kg; lane 7, (±)-NBP 20 mg/kg. (C) Semiquantitative analysis of relative abundance of cytochrome c immunoreativity determined by densitometric measurement. Cytochrome c levels are expressed as % of *OD* levels of the vehicle group (% of vehicle). *n*=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* vehicle. $^{f}P<0.01$ *vs* sham.

sistent (Fig 5A to 5B).

Cytosolic expression of cytochrome c was also analyzed by immunohistochemistry. There was no immunoreactivity in the sections of sham-operated rats (Fig 2D) or in the negative control sections (Fig 2E), which were treated without primary antibody. The absence of immunoreactivity is considered to be caused by thorough fixation of the brain by paraformaldehyde, which prevents the antibody from reaching the mitochondrial intermembrane space, but not the cytosol[22]. The massive increase of cytoplasmic cytochrome c immunoreactivity in vehicle treated rats (Fig 2F) at 24 h after reperfusion demonstrated the release of cytochrome c into the cytosol. The population of immunoreactive cells as well as the intensity of cytoplasmic immunostaining was markedly reduced in *s*-(-)-NBP (5, 10 mg/kg)-treated rats (Fig 2G, Tab 1), whereas the higher dose of *r*-(+)-NBP (10 mg/kg) showed a weaker effect, and the inhibitory effect of (±)-NBP (20 mg/kg) was in the range between that of *s*-(-)-NBP (10 mg/kg) and *r*-(+)-NBP (10 mg/kg), a profile similar to that obtained by Western blot analysis (Tab 1). Moreover, rats treated with *s*-(-)-NBP showed less immunostaining of the background, and some cells were accompanied by a long, axon-like structure protruding from the cytosol (arrows in Fig 2G). This might reflect the improving effect of *s*-(-)-NBP on the integrity of cells.

Tab 1. Effects of chiral NBP on expression of cytoplasmic cytochrome c protein and caspase-3 protein as measured by immunohistochemistry in MCAO (2 h of ischemia and 24 h of reperfusion) rats. -, no expression; +, weakly detectable expression; ++, prominent expression; +++, intense expression. *n*=3.

Group	Dose/ mg·kg^{-1}	Intensity of immunostaining Cytochrome c	Caspase 3
Sham		–, –, –	–, –, –
Negative control		–, –, –	–, –, –
Vehicle		+++, +++, +++	+++, +++, +++
s-(-)-NBP	5	++, ++, +++	++, ++, +++
	10	+, +, +	+, ++, ++
r-(+)-NBP	5	+++, +++, +++	+++, ++, +++
	10	++, ++, +++	++, +++, +++
(±)-NBP	20	+, ++, ++	++, ++, ++

Effects of chiral NBP on activation of caspase-3 Normally, caspase-3 protease is synthesized in cells as an inactive precursor (M_r=32000), which is cleaved into a small prodomain and two subunits of p17 (M_r=17000) and p12 (M_r=12000) when activated. The antibody we used recognized both the precursor and the p17 but not the p12 in the brain protein extracts (Fig 6). Brains from sham-operated rats showed high levels of precursor but none or very low levels of p17 subunit (Fig 6A to 6B, Lane 1). The precursor levels decreased and the p17 subunit appeared at 3 h after reperfusion (Fig 6A) and peaked at 24 h after reperfusion (Fig 6A). Therefore, the effects of chiral NBP were investigated at 24 h after reperfusion (Fig 6B to 6C). Similar to the effects on cytochrome c, *s*-(-)-NBP (5,

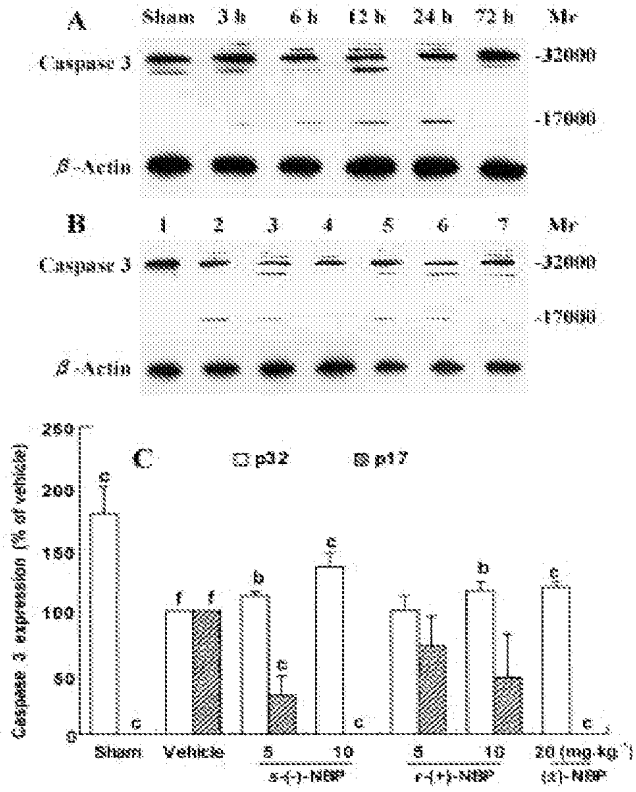


Fig 6. Western blot analysis of the procaspase-3 (M_r=32000) and its active fragment (M_r=17000) in MCAO rats with β-actin protein as an internal control. (A) Time-dependent changes in caspase-3 protein during reperfusion after 2 h of MCAO. (B) Effects of chiral NBP on the levels of caspase-3 after 24 h of reperfusion. Lane 1, sham; Lane 2, vehicle; Lane 3 and 4, *s*-(-)-NBP 5 and 10 mg/kg; Lane 5 and 6, *r*-(+)-NBP 5 and 10 mg/kg; Lane 7, (±)-NBP 20 mg/kg. (C) Semiquantitative analysis of relative abundance of caspase-3 immunoreactivity determined by densitometric measurement. Caspase-3 levels were expressed as % of *OD* levels of the vehicle group (% of vehicle). *n*=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* vehicle. $^fP<0.01$ *vs* sham.

10 mg/kg) (Fig 6B, Lane 3, 4) markedly inhibited ischemia-induced activation of caspase-3, whereas only *r*-(+)-NBP (10 mg/kg) (Fig 6B, Lane 6) showed a weaker effect (Fig 6C). The inhibitory effect of (±)-NBP (20 mg/kg) (Fig 6B, Lane 7) was shown to be in the range between that of *s*-(-)-NBP (10 mg/kg) and *r*-(+)-NBP (10 mg/kg) (Fig 6C).

With immunohistochemistry, remarkable immunoreactivity of caspase-3 was observed in the ischemic area in vehicle-treated rats (Fig 2J). However, in the sections of sham-operated rats (Fig 2H) or in the negative control slides (Fig 2I) incubated with the secondary antibody, immunoreactivity of caspase-3 was weak and diffusely expressed, and densely labeled caspase-3-positive cells were not detected. These results were consistent with previous reports[23-25]. As a confirmation of Western blot analysis, the increase in caspase-3 immunoreactivity markedly diminished in *s*-(-)-NBP (5, 10 mg/kg)-treated group (Fig 2K). However, a higher dose of *r*-(+)-NBP (10 mg/kg) caused only a marginal decrease and (±)-NBP (20 mg/kg) led to a reduction between that of *s*-(-)-NBP (10 mg/kg) and *r*-(+)-NBP (10 mg/kg) (Tab 1).

DISCUSSION

In this study, we demonstrated that *s*-(-)-, *r*-(+)- and (±)-NBP inhibited transient focal cerebral ischemia-induced apoptosis in rats. In addition, we found that this inhibitory effect was mainly due to *s*-(-)-NBP.

The involvement of apoptosis in the progression of tissue damage resulting from cerebral ischemia is currently intensively discussed[26-28], especially for the pathological situation of a transient obstruction of MCA, which commonly occurs in ischemic human stroke. Li *et al*[13] found that DNA fragmentation was initiated within 0.5 h after MCAO, peaked at 24-48 h, and persisted at least for 4 weeks, and most cells exhibiting DNA fragmentation were neurons. In the current study, a significant amount of DNA fragmentation was detected at 24 h affer reperfusion, as demonstrated by TUNEL staining and DNA laddering, which was consistent with previous reports[13]. The results also showed that NBP could markedly reduce DNA laddering and decrease the number of TUNEL-positive cells after transient focal cerebral ischemia. Although it did not prove the hypothesis that NBP directly inhibited apoptosis, the results of our present study at least suggested that the anti-ischemic effects of NBP might partially due to the reduction of apoptotic cell death. Because multiple pathways were involved in the apoptotic cascade after cerebral ischemia, mechanisms of the inhibitory effects of NBP seemed to be complicated. However, several important findings should be noted[9]. First, no change was found in mean arterial blood pressure and head temperature in NBP-treated rats compared with vehicle-treated rats. Second, regional cerebral blood flow could be markedly enhanced after NBP treatment[9]. Therefore, the improvement of regional cerebral blood flow might be one of the upstream events of the antiapoptotic effect of NBP.

There is evidence that the mitochondrial release

of cytochrome c and the subsequent activation of caspase-3 play the key role in cerebral ischemia-induced apoptosis[22]. Our results showed that after 2-h MCAO and 3-h reperfusion, the release of cytochrome c from mitochondria occurred, which peaked after 24-h reperfusion and sustained until 72 h after reperfusion. This was in concordance with previous reports[22]. We also detected the activation of caspase-3 in the ischemic zone as early as 3 h after reperfusion, with a maximal at 24 h. Therefore, in this study the time-point of 24 h after reperfusion was selected to investigate the effects of drugs. As demonstrated by Western blot analysis and immunohistochemistry, the release of cytochrome c and the activation of caspase-3 could be reduced by NBP. Although the relationship between the inhibitory effects of NBP on cerebral ischemia-induced cytochrome c release, caspase-3 activation and the inhibitory effects of NBP on cerebral ischemia-induced apoptosis required further study, our results at least provided the possibility that NBP might partially suppress apoptosis by inhibiting cytochrome c release and preventing caspase-3 activation after transient focal cerebral ischemia.

Our results revealed that *s*-(-)-NBP more potently inhibited apoptosis than *r*-(+)-NBP. The different actions of chiral enantiomers demonstrated the existence of stereoselectivity between biological macromolecule and small drug molecules. Interestingly, after treatment with (±)-NBP 20 mg/kg, which actually contained equal quantity of *s*-(-)- and *r*-(+)-NBP, the effect was much less apparent than that after treatment with *s*-(-)-NBP 10 mg/kg. Similar results were observed in the analysis of cytochrome c and caspase-3. This strongly suggested a possible antagonistic mechanism between *s*-(-)- and *r*-(+)-NBP, and more extensive studies are required. These experiments might provide important information for the study of correlation between optical stereoselectivities and pharmacological activities in drug development.

Taken together, NBP, especially its *s*-(-)-enantiomer, when administered after ischemia, potently reduced the release of cytochrome c, decreased the activation of caspase-3, and inhibited DNA fragmentation induced by transient focal cerebral ischemia. Our findings might have important implications for the study and treatment of ischemic cerebrovascular diseases.

ACKNOWLEDGMENT We thank Prof FENG Yi-Pu for giving us valuable directions in the experiments and Prof SONG Zhen-Yu for reading the manuscript.

REFERENCES

1 Yang JS, Chen YW. Isolation and identification of the anticonvulsive constituents of *Apium graveolens*. Chin Pharmacol Bull 1984; 19: 670-1.
2 Liu XG, Feng YP. Protective effect of *dl*-3-*n*-butylphthalide on ischemic neurological damage and abnormal behavior in rats subjected to focal ischemia. Acta Pharm Sin 1995; 30: 896-903.
3 Yao CH, Feng YP, Zhang JT. Effect of *dl*-3-*n*-butylphthalide on delayed neuronal damage after focal cerebral ischemia and intrasynaptosomes calcium in rats. Acta Pharmacol Sin 1998; 19: 117-20.
4 Xiong J, Feng YP. Effects of butylphthalide on the activities of complexes of the mitochondrial respiratory chain. Acta Pharm Sin 1999; 34: 241-5.
5 Xiong J, Feng YP. The protective effect of butylphthalide against mitochondrial injury during cerebral ischemia. Acta Pharm Sin 2000; 35: 408-12.
6 Feng YP, Hu D, Zhang LY. Effect of *dl*-butylphthalide (NBP) on mouse brain energy metabolism in complete brain ischemia induced by decapitation. Acta Pharm Sin 1995; 30: 741-4.
7 Deng WB, Feng YP. Effect of *dl*-3-*n*-butylphthalide on brain edema in rats subjected to focal cerebral ischemia. Chin Med Sci J 1997; 12: 102-6.
8 Chong ZZ, Feng YP. *dl*-3-*n*-Butylphthalide attenuates reperfusion-induced blood-brain barrier damage after focal cerebral ischemia in rats. Acta Pharmacol Sin 1999; 20: 696-700.
9 Yan CH, Feng YP, Zhang JT. Effects of *dl*-3-*n*-butylphthalide on regional cerebral blood flow in right middle cerebral artery occlusion rats. Acta Pharmacol Sin 1998; 19: 117-20.
10 Chong ZZ, Feng YP. Protective effects of *dl*-3-*n*-butylphthalide on changes of regional cerebral blood flow and blood-brain barrier damage following experimental subarachnoid hemorrhage. Chin Med J 1998; 111: 858-60.
11 Xu HL, Feng YP. Effects of 3-*n*-butylphthalide on production of vasoactive substances by cerebral and aortic endothelial cells. Acta Pharmacol Sin 1999; 20: 929-33.
12 Zhang LY, Feng YP. Effect of *dl*-3-*n*-butylphthalide (NBP) on life span and neurological deficit in SHRsp rats. Acta Pharm Sin 1996; 31: 18-23.
13 Li Y, Chopp M, Jiang N, Yao F, Zaloga C. Temporal profile of *in situ* DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 1995; 15: 389-97.
14 Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 1993; 24: 2002-8.
15 Du C, Hu R, Csernansky CA, Hsu CY, Choi DW. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? J Cereb Blood Flow Metab 1996; 16: 195-201.
16 Dong GX, Feng YP. Hypoxia/hypoglycemia-induced apoptosis of rat cortical neurons is prevented by *dl*-3-butylphthalide. Acta Pharm Sin 1999; 34: 176-80.

17 Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 1996; 86: 147-57.
18 Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281: 1309-12.
19 Maier CM, Ahern KV, Cheng ML, Lee JE, Yenari MA, Steinberg GK. Optimal depth and duration of mild hypothermia in a focal model of transient cerebral ischemia: effects on neurologic outcome, infarct size, apoptosis, and inflammation. Stroke 1998; 29: 2171-80.
20 Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke 1986; 17: 472-6.
21 Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992; 119: 493-501.
22 Fujimura M, Morita-Fujimura Y, Murakami K, Kawase M, Chan PH. Cytosolic redistribution of cytochrome c after transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab 1998; 18: 1239-47.
23 Guegan C, Sola B. Early and sequential recruitment of apoptotic effectors after focal permanent ischemia in mice. Brain Res 2000; 856: 93-100.
24 Kim GW, Sugawara T, Chan PH. Involvement of oxidative stress and caspase-3 in cortical infarction after photothrombotic ischemia in mice. J Cereb Blood Flow Metab 2000; 20: 1690-701.
25 Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, *et al*. Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. J Neurosci 1998; 18: 4914-28.
26 Savitz SI, Rosenbaum DM. Apoptosis in neurological disease. Neurosurgery 1998; 42: 555-72.
27 Schulz JB, Weller M, Moskowitz MA. Caspases as treatment targets in stroke and neurodegenerative diseases. Ann Neurol 1999; 45: 421-9.
28 Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, *et al*. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 1998; 18: 3659-68.
29 Nagayama T, Lan J, Henshall DC, Chen D, O'Horo C, Simon RP, *et al*. Induction of oxidative DNA damage in the peri-infarct region after permanent focal cerebral ischemia. J Neurochem 2000; 75: 1716-28.